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(54) Title: NEWCASTLE DISEASE VIRUS INFECTIOUS CLONES, VACCINES AND DIAGNOSTIC ASSAYS

(57) Abstract

The invention relates to the process for generating infectious Newcastle disease virus (NDV) entirely from cloned full–length cDNA and to the use of vaccines and diagnostic assays generated with and derived from said process. The process offers the possibility to modify the NDV genome by means of genetic modification and allows the introduction of mutations, deletions, and/or insertions. The process can be used to modify the virulence of NDV, thereby generating new attenuated live vaccines with enhanced properties. The process can be used to modify the antigenic make–up of NDV, thus allowing the generation of live NDV marker vaccines which can be serologically distinguished from NDV field strains.

Title: Newcastle disease virus infectious clones, vaccines and diagnostic assays.

The invention relates to Newcastle disease virus infections of poultry.

Newcastle disease virus (NDV) is one of the most diverse and deadly avian pathogens. The almost simultaneous occurrence of Newcastle disease as an apparent new disease in several different geographical locations and the great variation in type and severity of the disease has caused some problems with nomenclature.

The disease has been termed pseudo fowl pest, pseudo poultry plague, avian pest, avian distemper and avian pneumoencephalitis. The importance of the disease is primarily due to the development of the poultry industry during the 20th Century into a highly efficient international industry which is dependent on intensive trade between countries.

It is generally assumed that the first outbreaks of Newcastle disease occurred in 1926 in Java, Indonesia, and in Newcastle-upon-Tyne, England (Kraneveld, 1926; Doyle, 1927). The name Newcastle disease was coined by Doyle as a temporary name to avoid a descriptive name that might be confused with other diseases. It later became clear that other less severe diseases were caused by viruses indistinguishable from NDV. In the US a relatively mild respiratory disease was termed avian pneumoencephalitis and was shown to be caused by NDV (Beach, 1944). Within a few years, numerous NDV isolations that caused extremely mild or no disease in chickens were made around the world.

The following methods have been implicated in the spread of the disease: 1) movement of live birds, feral birds, game birds, racing pigeons and commercial poultry; 2) movement of people and equipment; 3) movement of poultry products; 4) airborne spread; 5) contaminated poultry feed; 6) contaminated water; 7) incompletely inactivated or

heterogeneous vaccines. According to the OIE, Newcastle disease is a disease of poultry caused by a virus of avian paramyxovirus serotype 1 (APMV-1) which has an intracerebral pathogenicity index (ICPI) in day-old chicks of 0.7 or greater. Virulent virus can also be confirmed by the presence of multiple basic amino acids at the C-terminus of the F2 protein and F (phenylanine) at residue 117, the N-terminus of the F1 protein. Failure to demonstrate this amino acid sequence would require characterisation by ICPI tests. The word 'poultry' refers to domestic fowl, turkeys, guinea fowl, ducks, geese, quails, pigeons, pheasants, partridges and ratites that are reared or kept in captivity for breeding, the production of meat or eggs for consumption, or for restocking supplies of game.

According to Alexander (1988) three panzootics of Newcastle disease have occurred since the first recognition of the disease. The first represented the initial outbreaks of the disease and appears to have arisen in South-East Asia. Isolated outbreaks, such as the one in England in 1926, were chance introductions ahead of the mainstream which slowly moved through Asia to Europe.

A second panzootic appears to have begun in the Middle East in the late 1960's and reached most countries by 1973 The more rapid spread of the second panzootic was probably caused by the major revolution of the poultry industry with considerable international trade.

A third panzootic primarily affected domesticated birds such as pigeons and doves (Vindevogel and Duchatel, 1988). The disease apparently arose in the Middle East in the late 1970's. By 1981 it had reached Europe and then spread rapidly to all parts of the world, largely as a result of contact between birds at races and shows and the international trade in such birds.

Nowadays, Newcastle disease is still widespread in many countries of Asia, Africa, the Americas, and Europe. Only the countries of Oceania appear to be relatively free from the disease (Spradbrow, 1988).

NDV belongs to the order *Mononegavirales*, family *Paramyxoviridae*, subfamily *Paramyxovirinae*, genus *Rubulavirus*. Apart from NDV, generally called avian paramyxovirus type-1, eight other serotypes, designated avian paramyxovirus type-2 to -9, can be distinguished on the basis of their antigenic relatedness in hemagglutination-inhibition tests and serum neutralisation tests (Alexander, 1993).

Despite the consistency of the serological grouping there are some cross-relationships between viruses of the different serotypes.

The genome of NDV is a single-stranded RNA molecule of negative polarity, complementary to the messenger RNA's which code for the virus proteins. The RNA genome is approximately 15,200 nt in size and codes for the following gene products (listed from the 3' end to the 5' end of the genomic RNA): nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and large polymerase protein (L) (Chambers et al., 1986).

The RNA is complexed with the NP, P and L proteins to form a ribonucleocapsid particle (RNP) that is surrounded by an envelope that is lined at the inside by the M protein. The envelope contains the F and HN proteins which are involved in attachment and penetration of the host cell.

Replication of NDV is similar to the strategy used by other paramyxovirinae. The initial step is attachment of the virus to the host cell receptors, mediated by the HN protein. Fusion of the viral envelope with the host cell membrane is dependent on the action of both the HN and F proteins and results in the release of the RNP into the cytoplasm where virus replication takes place.

The viral RNA-dependent RNA polymerase (which is part of the RNP) produces complementary transcripts that act as mRNA's and are used by the cell's translation machinery for the synthesis of virus proteins. Due to the accumulation of NP protein, the RNA polymerase complex switches from

transcription to replication, resulting in the synthesis of full-length genomic and antigenomic RNA molecules.

Newly formed RNP's are encapsidated at the cellular membrane by the action of the M protein and the F and HN proteins which have accumulated in the cellular plasma membrane. Newly formed virus particles are released from the infected cell by a budding mechanism. For more detailed information about NDV replication see Peeples (1988). For a recent review of the molecular biology of paramyxovirinae see Lamb and Kolakofsky (1996).

Apart from commercial domestic poultry (i.e. chickens, turkeys, pheasants, guinea fowl, ducks, geese, pigeons), a wide range of captive, semi-domestic and free-living birds, including migratory waterfowl, is susceptible to NDV and can be primary infection sources (Kaleta and Baldauf, 1988).

The pathogenicity of NDV strains differs greatly with the host. The most resistant species appear to be aquatic birds while the most susceptible are gregarious birds forming temporary of permanent flocks. Chickens are highly susceptible but ducks and geese may be infected and show few or no clinical signs, even with strains which are lethal for chickens.

Newcastle Disease is complicated in that different isolates and strains of the virus may induce enormous variation in the severity of the disease. Beard and Hanson (1984) grouped NDV strains and isolates into different pathotypes that relate to disease signs that may be seen in fully susceptible chickens: 1) viscerotropic velogenic NDV, which produces acute lethal infections in which hemorrhagic lesions are prominent in the gut; and neurotropic velogenic NDV, which produces high mortality preceded by respiratory and neurological signs, but no gut lesions; 2) mesogenic NDV, which produces low mortality, acute respiratory disease and nervous signs in some birds; 3) lentogenic NDV, which produces mild or inapparant respiratory infections or even asymptomatic enteric NDV, avirulent viruses that appear to replicate primarily in the intestinal tract. Some overlap

between the signs associated with the different groups has been reported.

The virus enters the body via the respiratory and the intestinal tract or via the eye. In the trachea, the virus is spread by ciliary action and by cell-to-cell spread. After initial multiplication at the introduction site, virus is carried during episodes of viraemia to spleen, liver, kidney and lungs. Viruses of some strains reach vital organs like liver and kidney very rapidly so that the birds may die before disease symptoms are overt.

Most viruses reach the central nervous system via the blood before significant amounts of antibody exist. A long, asymptomatic carrier state presumed to occur in psittacines constitutes a potential threat to the poultry industry. A long term carrier state of both lentogenic and velogenic virus may also exist in chickens (Heuschele and Easterday, 1970).

During the replication of NDV it is necessary for the precursor glycoprotein Fo to be cleaved to F1 and F2 for the progeny virus to be infectious (Rott and Klenk, 1988). This posttranslational cleavage is mediated by host cell proteases. If cleavage fails to take place, non-infectious virus particles are produced and viral replication cannot proceed. The Fo protein of virulent viruses can be cleaved by a wide range of proteases, but Fo proteins in viruses of low virulence are restricted in their sensitivity and these viruses can only grow *in vivo* in certain host cell types and in general cannot be grown *in vitro*.

Lentogenic viruses only replicate in areas with trypsin-like enzymes such as the respiratory and intestinal tract, whereas virulent viruses can replicate in a range of tissues and organs resulting in fatal systemic infection.

Amino acid sequencing of the Fo precursor has shown that low-virulence viruses have a single arginine (R) that links the F2 and F1 chains, whereas virulent strains possess additional basic amino acids forming two pairs such as

K/R-X-K/R-R-F at the site of cleavage. Furthermore, the F2 chain of virulent strains generally starts with a phenylalanine residue whereas that of nonvirulent strains generally starts with a leucine.

For a few strains of NDV the HN protein is also produced as a precursor that requires cleavage to be biologically active (Garten et al., 1980; Millar et al., 1988).

Besides cleavability of the F and HN proteins, other viral factors may contribute to pathogenicity. Madansky and Bratt (1978, 1981a, 1981b) have shown that alterations in transcription and translation could modulate growth and cell-to-cell spread of the virus and/or cytopathogenicity.

The initial immune response to infection with NDV is cell mediated and may be detectable as early as 2-3 days after infection with live vaccine strains. This presumably explains the early protection against challenge that has been recorded in vaccinated birds before a measurable antibody response is seen (Gough and Alexander, 1973).

At about 1 week after infection, circulating antibodies may protect the host from re-infection. In the early phase IgM is involved, followed by IgG. Titres and protection peak after about 3 weeks and gradually decline if there is no boosting. This means that for older birds, re-vaccinations are necessary.

Only live vaccines administered by the respiratory route stimulate antibody in all mucosal surfaces as well as in serum. Inactivated vaccine, even when applied via the intramuscular route, does not elicit local resistance in the respiratory tract, despite high concentrations of serum antibody.

This stresses the importance of live vaccines capable of presenting viral antigen to the upper respiratory tract to induce both local and systemic immunity. Small droplets penetrate into the lower respiratory tract thereby provoking

a mainly humoral immune response, while coarse droplets stimulate local immunity in the upper respiratory tract.

Therefor, aerosols with a wide range of droplet sizes generate the best overall local and humoral immunity.

It should be noted, however, that despite intensive vaccination with current vaccines creating high levels of antibody titers, virus can still be recovered from mucous surfaces.

The identification of Newcastle disease in the USA led to the use of inactivated vaccines (Hofstad, 1953). The observation that some of the enzootic viruses produced only mild disease resulted first in the development of the mesogenic live vaccine Roakin (Beaudette et al., 1949) and, subsequently, in the development of the milder Hitchner B1 (Hitchner and Johnson, 1948) and LaSota (Goldhaft, 1980) strains, which are now the most widely used live vaccines.

NDV live vaccines can be divided into two groups, lentogenic and mesogenic. Mesogenic strains are suitable only for secondary vaccination of birds due to their greater virulence. The immune response increases as the pathogenicity of the live vaccine increases. Therefore, to obtain the desired level of protection without serious reaction, currently vaccination programs are used that involve sequential use of progressively more virulent vaccines, or live vaccines followed by inactivated vaccines.

On of the main advantages of live vaccines is that they may be administered by inexpensive mass application techniques. A common method of application is via drinking water. However, drinking water application must be carefully monitored as the virus may be inactivated by excessive heat and light and by virucidal impurities in the water.

Mass application of live vaccines by sprays and aerosols is also very popular due to the ease with which large numbers of birds can be vaccinated in a short time. It is important to achieve the correct particle size by controlling the conditions under which the particles are generated.

Currently used live vaccines have several disadvantages. The vaccine may still cause disease signs, depending upon environmental conditions and the presence of complicating infections. Therefore, it is important to use extremely mild virus for primary vaccination and, as a result, multiple vaccinations are usually needed. Furthermore, maternally derived antibodies may prevent successful primary vaccination with lentogenic live vaccines.

Inactivated vaccines are usually produced from infectious allantoic fluid which is treated with formalin or betapropiolactone to kill the virus and mixed with a suitable adjuvant. Inactivated vaccines are administered by injection, either intramuscularly or subcutaneously. Inactivated vaccines are expensive to produce and to apply.

However, inactivated oil-emulsion vaccines are not as adversely affected by maternal immunity as live vaccines and they can be used in day-old chicks. Advantages of inactivated vaccines are the low level of adverse reactions in vaccinated birds, the high level of protective antibodies, and the long duration of protection. None of the above vaccines can serologically be differentiated from wild-type NDV.

The development of recombinant viral vaccines has been of interest to the poultry industry for a number of years. The concept is to insert genes of critical immunising epitopes of a disease agent of interest into a nonessential gene of a vector virus. Vaccination with the recombinant virus thus results in immunisation against both the vector virus as well as the disease agent of interest.

Several types of viruses have been evaluated as potential live viral vaccines for poultry. Two avian viruses that have received most attention are fowlpox virus (FPV) and herpesvirus of turkeys (HVT). Fowlpox virus is a DNA virus that has a large genome and hence is considered to have ample room to carry foreign genes.

When attenuated, FPV does not cause clinical disease and is commonly used as a vaccine in chickens. HVT is also a DNA

virus and is classified as serotype III of the Marek's disease virus (MDV) family. HVT is non-pathogenic for chickens yet cross-protective against MDV and is commonly used to vaccinate chickens against Marek's disease.

It has been shown that protection against Newcastle disease can be induced by using recombinant HVT or FPV vaccines (Morgan et al., 1992, 1993; Heckert et al., 1996; Boursnell et al., 1990; Taylor et al., 1990).

However, the onset of protection against Newcastle disease following vaccination with such recombinant vaccines that express either the NDV F protein or both the F and HN proteins was severely delayed compared to that following vaccination with a conventional live or inactivated NDV vaccine, possibly because the recombinant vaccines do not provide a wide enough immunological spectre of antigenically relevant NDV epitopes other than those found on the NDV protein that is expressed by the recombinant vaccine or are not properly presented to the immune system.

Furthermore, local (mucosal, respiratory or enteric) protection was not effectively induced in birds vaccinated with the recombinants. This is a serious drawback since vaccines used for primary vaccination against respiratory diseases must induce local immunity to prevent infection and spread of virulent viruses that infect chickens reared under field conditions.

Antibodies against NDV which are capable of protecting the host can be measured in virus neutralisation tests. However, since the neutralisation response appears to parallel the haemagglutination inhibition (HI) response, the latter test is frequently used to assess the protective response, especially after vaccination.

Antibodies against both the F and HN proteins can neutralise NDV. However, antibodies against the F protein appear to induce greater neutralisation than those directed against HN in *in vivo* and *in vitro* tests (Meulemans et al., 1986).

The presence of specific antibodies to NDV in the serum of a bird gives little information on the infecting strain of NDV and therefore has limited diagnostic value.

The omnipresence of lentogenic NDV strains in birds in most countries and the almost universal use of live vaccines that can not be distinguished, at least not serologically from wild-type NDV, means that the mere demonstration of infection is rarely adequate cause for control measures to be imposed. Since field disease may be an unreliable measure of the true virulence of the virus, it is necessary to further characterise the virus that is found.

At present, the only method of Newcastle disease diagnosis which allows characterisation of the infecting strain, is virus isolation followed by pathogenicity testing. At present, three *in vivo* tests are used for this purpose: 1) mean death time (MDT) in eggs; 2) Intracerebral pathogenicity index (ICPI) in one-day-old chickens; 3) Intravenous pathogenicity index (IVPI) in 6-week-old birds.

These tests suffer from a number of drawbacks, such as the availability of animals, poor reproducibility, and the relatively long duration of the tests. Last but not least, these tests do not allow a simple serological identification of poultry vaccinated with a vaccine or infected with a wild-type strain.

As an alternative to *in vivo* tests, the polymerase chain reaction (PCR) has been successfully used to distinguish between virulent and non-virulent i and non-virulent isolates (Staüber et al., 1995; Kant et al., 1997), however, again serological differentiation is not possible.

The raising of poultry and trade of their products is now organised on a international basis, frequently under management of multinational companies. The threat of Newcastle disease has proven a great restraint on such trade.

Successful control of Newcastle disease will only be approached when all countries report outbreaks. However,

international agreements are not simple due to enormous variation in the extent of disease surveillance in different countries. Some countries do not vaccinate and would not want any form of NDV introduced in domestic poultry because vaccinated poultry cannot be distinguished from those infected with wild-type NDV.

Others only allow the use of specific live vaccines and consider other vaccines as unacceptably virulent. Yet other countries have the continued presence of circulating highly virulent virus, which is not recognised as such because overt disease is masked by vaccination.

In many countries legislation exists to control Newcastle disease outbreaks that may occur. National control measures are directed at prevention of introduction and spread. Most countries have restrictions on trade in poultry products, eggs, and live poultry. Most countries have established quarantine procedures for importation, especially for psittacine birds.

Some countries have adopted eradication policies with compulsory slaughter of infected birds, their contacts, and products. Others require prophylactic vaccination of birds even in the absence of outbreaks, while some have a policy of ring vaccination around outbreaks to establish a buffer zone.

Clearly, there is a need for better vaccines and for better diagnostic methods which can be used to control Newcastle disease. Due both to large differences in the dose that is received by individual birds during mass application of live vaccines and to variation in levels of maternal immunity in young chickens, post-vaccination reactions with live vaccines are inevitable. This is one of the main concerns of farmers in countries where vaccination is compulsory.

Furthermore, many vaccines are mixtures of sub-populations. When cloned, these sub-populations may differ significantly from each other in immunogenicity and pathogenicity (Hanson, 1988).

However, the largest drawback of currently used live vaccines and inactivated vaccines is the fact that vaccinated animals cannot be distinguished from infected animals with currently used screening techniques such as heamagglutination-inhibition or virus neutralisation tests.

Virulent field-virus may still spread in vaccinated flocks since disease symptoms are masked by vaccination. Since virus isolation and characterisation of virulence by *in vivo* techniques is not feasible on a large scale, there is a great need for new and effective attenuated live vaccines which can be serologically discriminated from field-viruses.

Such vaccines, called NDV marker vaccines (and accompanying diagnostic methods and kits) , which should provide the fullest possible immunological spectre of antigenically relevant NDV epitopes, and yet should be serologically distinct from wild-type NDV are not yet available.

The invention provides a method to modify an avian-paramyxovirus genome by genetic modification, provides genetically modified avian-paramyxovirus and an avian-paramyxovirus marker vaccine.

The advent of modern molecular biological techniques has allowed the genetic modification of many RNA viruses, including negative-strand RNA viruses. This technique is often referred to as "reverse genetics". One first provides a (full-length) cDNA copy of the viral RNA, after which one transcribes this DNA in susceptible cells to produce infectious RNA which can again replicate to produce infectious virus particles.

In general, by previous modification of the cDNA with standard molecular biological techniques, it is possible to obtain a genetically modified RNA virus. However, this has never materialised for NDV or other avian-paramyxoviruses, it has even not yet been possible to generate minigenome

fragments or plasmids of avian-paramyxovirus genomic fragments to study replicative events of avian-paramyxovirus, thereby creating an understanding on how to construct infectious copy virus.

Surprisingly, although in this description it has now been fully established that the genome of avian-paramyxovirus is the smallest of all paramyxovirus genomes sequenced up to now, especially the 5' terminal end sequence of the NDV genome is much longer than previously had been established and was expected by comparison with other *Paramyxoviridae*. The invention now for the first time provides a full sequence of an avian-paramyxovirus genome and provides full-length or minigenomic length cDNA of such a virus.

The invention herewith provides avian-paramyxovirus cDNA at least comprising a nucleic acid sequence corresponding to the 5'-terminal end of the genome of avian-paramyxovirus allowing generating an infectious copy of avian-paramyxovirus, said cDNA preferably comprising a full-length cDNA. However, the invention also provides cDNA at least comprising a nucleic acid sequence corresponding to the 5'-terminal end of the genome of avian-paramyxovirus thereby allowing generating an replicating avian-paramyxovirus minigenome. Such minigenomes can advantageously be used to transcribe RNA and/or express protein from modified nucleic acid sequences. The invention provides a cDNA according to the invention at least partly derived from Newcastle Disease Virus, for example wherein said Newcastle Disease Virus is a lentogenic virus, preferably derived from a vaccine strain, such as LaSota strain ATCC VR-699.

The invention furthermore provides a cDNA according to the invention additionally provided with a modification, such as a deletion, insertion, mutation, reversion, or otherwise in a nucleic acid. For example a cDNA is provided wherein said modification comprises a nucleic acid encoding a modified protease cleavage site, for example wherein said

cleavage site is a protease cleavage site of the fusion (F) protein.

In yet another embodiment, the invention provides a cDNA according to the invention wherein said modification comprises a nucleic acid encoding a hybrid viral protein, such as a hybrid hemaglutinin-neuraminidase (HN) protein as described in the experimental part of the invention. The invention also provides a cDNA according to the invention wherein said modification comprises a deletion in a nucleic acid encoding a viral protein, such as a matrix (M) protein.

The invention additionally provides a cDNA according to the invention additionally provided with a nucleic acid encoding an heterologous antigen, preferably wherein said antigen is derived from a poultry pathogen, as for example described below. An RNA, and protein derived thereof, obtained from a cDNA according to the invention is also provided.

In recent years, a number of non-segmented negative-strand RNA viruses has been fully characterised and fundamental work on the replication and expression of their genomes has culminated in the ability to generate infectious virus entirely by transfecting cells with cloned cDNA of said virus (reviewed by Conzelmann, 1996).

To date, infectious virus of non-segmented negative-strand RNA viruses has been generated from cloned cDNA of for example rabies virus (Schnell et al., 1994, Conzelmann; EP0702085A1). (Schnell et al., 1994; EP0702085A1), vesicular stomatitis virus (Lawson et al., 1995; Whelan et al., 1995), Sendai virus (Garcin et al., 1995), measles virus (Radecke et al., 1995; Schneider et al., 1997; EP0780475A1), human respiratory syncytial virus (Collins et al., 1995), rinderpest virus (Baron and Barrett, 1997), and human parainfluenza virus type 3 (Hoffman and Banerjee, 1997, Conzelmann; P0702085A1), (Schnell et al., 1994; EP0702085A1).

However, all of above infectious copy viruses are capable of growing both *in vivo* as well as *in vitro* in hosts,

tissues or cells of various origin, allowing easy cDNA transfection and replication and generation of infectious virus particles on a suitable cell line.

Such possibility does not exist for NDV, certainly not for lentogenic NDV strains which can provide a vaccine. Virulence of such an NDV strain is associated with its ability to replicate in a wide range of cells, reflected by the fact that virulent strains can easily replicate *in vitro* and *in vivo*, whereas vaccine strains can only replicate *in vivo*.

Thus, with NDV a catch 22 situation is apparent. While attempts to generate an infectious copy virus from for example infectious cDNA may possibly result in infectious virus, such virus is in general not suitable for use as a vaccine because the thus generated infectious virus is by default too virulent to be used as vaccine; the fact that it can be generated and replicated after transfection of cDNA on a cell line reflects its easy cleavability of the Fo protein into F1 and F2, as discussed above a hallmark of virulence of a NDV.

Using a vaccine strain as parent material for the cDNA would not solve this problem; a vaccine strain, especially of a lentogenic type does not contain an easily cleavable Fo protein, rendering it impossible for first generation virus to continue to replicate. The cell used for transfection will simply not be susceptible to support one or more rounds of replication of vaccine-type virus with a non-cleaved Fo protein.

The invention now elegantly provides a solution for this problem, and therewith provides infectious copy NDV, for example for use in a vaccine.

The invention provides a method to generate infectious copy Newcastle Disease Virus comprising transfecting cells, capable of expressing viral NP, P and L proteins for complexing with viral RNA with cloned full-length or genomic-length cDNA of said virus and further comprising incubating

said cells in growth medium comprising proteolytic activity allowing cleavage of the Fo protein of said virus.

In our system, co-transfection of a plasmid expressing NP could be omitted. NP is probably expressed from the full length cDNA because the NP gene is the first gene after the 5' end of the antigenomic RNA. Since eukaryotic mRNA's are usually monocistronic, expression of distal genes is not expected. However it is possible to generate full-length cDNA in which the relative positions of the NDV genes are changed. If the first gene of such a cDNA is the P or L gene, it is not necessary to express the corresponding gene product from a co-transfected plasmid.

As an alternative to using full-length cDNA, it is possible to use two or more subgenomic cDNA's which generate replication competent subgenomic RNA's and which together express the full complement of avian-paramixovirus proteins. Even if the RNA's are packaged separately, the resulting virus-like particles can be used for successive rounds of replication by means of co-infection and complementation of gene functions.

In a preferred embodiment, the invention provides a method wherein said proteolytic activity is derived of an enzyme, such as a trypsin-like enzyme, or is derived of a composition comprising said proteolytic activity. In a much preferred embodiment, said growth medium comprises allantoic fluid comprising proteolytic activity. Cleavage of the Fo protein is required for the generation of infectious virus. It is possible to generate infectious virus from lentogenic strain without the addition of exogenous proteolytic activity. By inoculating the supernatant of transfected cells into the allantoic cavity of embryonated eggs, the proteolytic activity which is present in the allantoic fluid is able to cleave the FO protein to generate the fusion-competent F1-F2 complex. Virions with such an activated F protein are able to infect susceptible cells and replication in cells which express the desired proteolytic activity yields infectious progeny. As an alternative to providing the

desired proteolytic activity to the supernatant of transfected cells, it is for example possibe to use a cell that is permissive for NDV and which already expresses said proteolytic activity. Such a cell line is used to produce infectious lentogenic NDV without the addition of exogenous proteolytic activity. Such a cell line can also be generated by stable transfecting a cell line with a gene that specifies said activity. Furthermore, it is possible to generate a stable transfected cell line that expresses the wild-type F protein in the virus envelope, thereby providing infectious particles (themselves not provided with genomic information encoding wild-type F protein) with means to enter a cell. Rescue of infectious lentogenic virus is also possible by infection of transfected cells with an NDV helpervirus. An essential requirement for such a helpervirus would be that it can be selected against, for instance by means of neutralizing antibodies which eliminate the helpervirus but which do not react with the lentogenic virus.
Finally, one may construct a stably transfected cell line that expresses one, two, or all of the three essential NDV proteins, NP, P, and L. Such cell lines require the co-expression of only a subset of the three essential proteins or no co-expression at all for supporting generating infectious copy virus.

In a preferred embodiment, the invention provides a method wherein said cells used for transfecting are derived of chicken primary or secondary cells or cell-lines. The description provides for example CER or CEF cells, which, as most *in vitro* cells in general, lack the appropriate proteases which are required to cleave the Fo protein of NDV, for example of strain LaSota. However, cells derived from for example other birds can also be used.

The invention further provides a method to generate infectious copy Newcastle Disease Virus comprising transfecting cells with cloned full-length or genomic-length cDNA of said virus as for example identified in figure 3 and

further comprising incubating said cells in growth medium comprising proteolytic activity allowing cleavage of the Fo protein of said virus, further comprising recovering infectious virus by culturing said cells and inoculating material derived from said cultured cells into the allantoic cavity of embryonated eggs. Said material for example comprises (harvested or freeze-thawed) cells or cell debris or supernatant derived from said cell culture.

For example, the description describes a method to recover infectious virus, wherein the supernatant of transfected CEF monolayers was inoculated into the allantoic cavity of embryonated eggs. Four days later the allantoic fluid was harvested, analyzed in a haemagglutination assay, and passaged further in eggs.

In addition, the invention provides a method further comprising passaging said infectious copy Newcastle Disease Virus by harvesting allantoic fluid and re-inoculating embryonated eggs.

In a preferred embodiment of the invention, a method is provided wherein said virus is a lentogenic virus, for example derived from an avirulent field-case of NDV or from a vaccine strain of NDV, such as the LaSota strain of NDV. Furthermore, a method is provided to modify an avian-paramyxovirus genome by means of genetic modification which allows the introduction of one or more mutations, deletions, and/or insertions or other modifications. For example, method is provided to attenuate or modify the virulence of avian-paramyxovirus by modifing cDNA, for example encoding a viral protein, such as the V protein, and cloning said modified cDNA into full-length cDNA and generating infectious copy virus from said full-length cDNA, thereby generating new NDV strains or new attenuated live vaccines with improved properties.

Apart from attenuation by modification of gene products it is also possible to attenuate avian-paramyxovirus by modification of nucleotide sequences which are involved in transcription and/or replication. Such modifications result

in attenuated strains which express wild type like F proteins which are cleavable both *in vitro* and *in vivo* in a wide range of cells and as a result are more immunogenic than the classical vaccine strains.

In a preferred embodiment, the invention provides a method to attenuate or modify the virulence of an avian paramyxovirus such as a Newcastle Disease Virus, comprising modifying a protease cleavage site of a viral protein by modifying cDNA encoding said cleavage site, further comprising cloning said cDNA into genomic length cDNA of e.g. Newcastle disease virus and generating infectious copy Newcastle Disease virus. Said cleavage site is for example a protease cleavage site in the F or HN protein of Newcastle Disease Virus. Attenuation is in general restricted to reduction of virulence, however, it is now also possible to use a relatively a-virulent strain of NDV and provide the progeny of such a strain with increased virulence, for example by providing it with an increased tendency to replicate in a specified cell-type. It is now thus possible to assign distinct virulence attributes to NDV.

The invention provides a method to antigenically modify avian paramyxovirus such as a Newcastle Disease Virus, comprising modifying cDNA encoding at least a part of a viral protein harbouring at least one immunodominant epitope, further comprising cloning said cDNA into genomic length cDNA of Newcastle disease virus and generating infectious copy Newcastle Disease virus.

For example, the invention provides a method to (further) modify NDV, for example using a method to produce an infectious copy of NDV (vaccine) which has been provided, a method to produce a recombinant marker NDV vaccine is provided, a marker vaccine that contains the fullest possible or needed immunological spectrum of antigenically relevant NDV epitopes, and yet is serologically distinct from wild-type NDV because a distinct, serologically relevant epitope or marker has been removed by recombinant techniques. The

invention provides a method to modify the antigenic make-up of avian paramyxovirus such as NDV, thus allowing the generation of e.g a live NDV marker vaccine which can be serologically distinguished from avian paramyxovirus field strains.

In one embodiment, the invention provides infectious copy NDV wherein the HN protein of NDV has been modified by recombining cDNA encoding a part of said HN protein with cDNA encoding a part of HN protein derived from an avian-paramyxovirus, for example type 2 or type 4. Said hybrid HN protein serves as a serological marker for the infectious copy NDV strain thus obtained or can serve to change the tropism of the avian paramyxovirus to other cells and/or tissues. These, so called, marker strains as provided by the invention allow the generation of vaccines which are an invaluable tool to assess the prevalence of NDV in commercial flocks around the world. Furthermore, the large-scale application of such marker vaccines will lead to the complete eradication of NDV by a process of intensive screening and stamping out of infected flocks.

Furthermore, a method is provided to generate an infectious copy NDV strain which expresses one or more antigens from other pathogens and which can be used to vaccinate against multiple diseases. Such an infectious copy NDV virus for example comprises a heterologous cDNA encoding a heterologous protein obtained from for example Avian Influenza (AI) (Haemagglutinim (H5 and H7) and Neuraminidase), Avian leukosis virus (ALV) (env protein (gp85)), Chicken anemia virus (CAV) (VP1+VP2), Marek's disease virus (MDV) (glycoprotein B (gB), gH), Infectious laringotracheitis virus (ILT) (gB, gH, gD), Infectious bursal disease virus (IBDV) (VP2 and VP3), Turkey rhinotracheitis virus (TRT) (fusion (F) protein), Avian paramyxovirus-2,-3,-6 (PMV) (F-protein, Haemagglutinin neuraminidase (HN), or others, Infectious bronchitis virus (IBV) (peplomer protein, nucleoprotein), Reoviruses (sigma protein), Adenoviruses Pneumoviruses, Salmonella enteritidis, Campylobacter

jejuni,Escherichia coli, Bordetella avium (formerly Alcaligenes faecalis), Haemphilus paragallinarum, Pasteurella multocida, Ornithobacterium rhinotracheale, Riemerella (formerly Pasteurella) anatipestifer, Mycoplasmata (M. gallisepticum, M synoviae, M. mereagridis, M. iowae), or Aspergilli (A. flavus, A. fumigatus).

The invention herewith provides avian-paramyxovirus or strains derived thereof which can be used as a vaccine vector for the expression of antigens from other poultry pathogens. Several properties make NDV an ideal vaccine vector for vaccination against respiratory or intestinal diseases. 1) NDV can be easily cultured to very high titres in embryonated eggs. 2) Mass culture of NDV in embryonated eggs is relatively cheap. 3) NDV vaccines are relatively stable and can be simply administered by mass application methods such as by drinking water or by spraying or aerosol formation. 4) The natural route of infection of NDV is by the respiratory and/or intestinal tract which are also the major natural routes of infection of many other poultry pathogens. 5) NDV can induce local immunity despite the presence of circulating maternal antibody.

It has been shown that NDV has potent antineoplastic, as well as immune-stimulating properties (for a review see Schirrmacher et al., 1998) [Schirrmacher, V., Ahlert, T., Steiner, H.-H., Herold-Mende, C., Gerhards, R. and Hagmüller E. (1998) Immunization with virus-modified tumor cells. *Seminars in Oncology* 25: 677-696]. Although NDV does not seem to be able to replicate productively in normal human cells, a selective NDV-mediated killing of human cancer cells was noted. After local NDV therapy, viral oncolysis and complete remissions of human tumor xenografts were observed in nude mice. This has led to the use of NDV for tumor therapy. However, a problem is that such application may be restricted to local treatment.

NDV infection induces interferons, chemokines, and other potentially important gene products, and introduces

pleiotropic immune-stimulatory properties into tumor cells. This concept has been used for the production of autologous tumor cell vaccines consisting of fresh operative specimens that have been infected with NDV. This type of vaccine is called autologous tumor vaccine-NDV or ATV-NDV (Schirrmacher et al., 1998). The NDV-infected cells are inactivated by gamma-irradiation which prevents cell division but which still allows replication of NDV in the cytoplasm of infected cells. After inoculation of patients with ATV-NDV, T-cells are recruited through NDV-induced chemokines. Some of these T-cells may express a T-cell receptor that can interact with peptides from tumor-associated antigens in complex with major histocompatibility complex class I molecules at the cell surface. This interaction results in the induction of a cytotoxic T-cell response which results in specific killing of autologous tumor cells.

The invention provides that the repertoire and amount of chemokines and immune stimulatory proteins induced by NDV infection are modulated. The present invention provides a method for generating recombinant NDV that has been modified to incorporate and express (a) heterologous gene(s). Such recombinant NDV may be used to modify the repertoire and amount of immune-stimulatory proteins in infected cells. In one embodiment, the invention provides a recombinant NDV that incorporates and expresses genes encoding human interferons, chemokines or other immune stimulatory proteins. Said recombinant NDV is used for the production of ATV-NDV which is more potent than conventional ATV-NDV. For example: cytokines IFN-α, -β, TNF-α, IL-1, IL-6; chemokines RANTES, IP-10; other genes such as HSP, ACTH, endorphin, iNOS, EPA/TIMP, NFκB.) The pleiotropic immune-stimulatory properties of NDV may also be used as an adjuvant for vaccination of animals and humans against infectious diseases. In one embodiment of the invention, foreign genes encoding (a) relevant antigen(s) of (an) infectious agent(s) are introduced in the NDV genome and the simultaneous expression of the antigen(s) and the immune-stimulatory

proteins by infected cells may induce a potent immune response against the infectious agent. In another embodiment of the invention, the immune-stimulating properties of NDV may be further enhanced by using NDV recombinants that simultaneaously express antigens and specific immune-stimulatory proteins. In a preferred embodiment, the invention is used to generate an AIDS (acquired immune-deficiency syndrome) vaccine by using NDV recombinants that express relevant antigens of human immune-deficiency virus (HIV), either alone or in combination with immune-stimulatory proteins.

NDV are also used as an adjuvant for vaccination of animals and humans against infectious diseases. In one embodiment of the invention, heterologous or foreign genes encoding (a) relevant antigen(s) of (an) infectious agent(s) are introduced in the NDV genome and the simultaneous expression of the antigen(s) and the immune-stimulatory proteins by infected cells may induce a potent immune response against the infectious agent. In another embodiment of the invention, the immune-stimulating properties of NDV are further enhanced by using NDV recombinants that simultaneaously express antigens and specific immune-stimulatory proteins. In a preferred embodiment, the invention is used to generate an AIDS (acquired immune-deficiency syndrome) vaccine by using NDV recombinants that express relevant antigens of human immune-deficiency virus (HIV), either alone or in combination with immune-stimulatory proteins.

Also, a method is provided to generate a conditional lethal NDV deletion mutant which can be used as self-restricted non-transmissible (carrier)vaccine. An NDV deletion mutant was generated which is unable to express the matrix (M) protein which is involved in budding of NDV at the inner cell membrane. The invention provides for example a phenotypically complemented NDV strain that is unable to express the M protein which is still able to infect cells and spread by means of cell-to-cell transmission. However, the

mutant virus is unable to generate infectious progeny on non-complementing cells. This shows that phenotypically complemented NDV deletion mutants can be used as safe self-restricted vaccines which are unable to spread into the environment. Such a non-transmissible vaccine combines the most important advantage of live vaccines, i.e., efficacy, with the most important advantage of killed vaccines, i.e., safety.

The invention provides Newcastle Disease Virus, or strains derived thereof, for example by passaging or further cultivation in embryonated eggs or appropriate cells, that is derived from infectious copy virus obtainable by a method provided by the invention.

For example, NDV is provided that has been modified in at least one way to generate infectious copy Newcastle Disease Virus which is attenuated, modified in virulence, antigenically modified, expressing a heterologous antigen or are non-transmissible, or combinations thereof.

Herewith the invention provides NDV vaccines, characterised for example by carrying distinct virulence attributes or distinct antigenic characteristics, be it for marker vaccine purposes and/or for expressing heterologous antigens derived from other pathogens, be it in transmissible and/or non-transmissible form.

Such a vaccine can be a killed or a live vaccine. Preferably, such a vaccine is a live vaccine, however, killed vaccines as provided by the invention are beneficial under those circumstances where a live vaccine is not or only little applicable, for example because of trade restrictions or other conditions set by disease controlling authorities.

The invention herewith also provides a diagnostic method, and corresponding test kit, to detect antibodies against said serologically relevant immunodominant epitope or marker, therewith providing methods and means to execute a method for control and/or eradication of NDV and/or other poultry diseases in poultry. The invention provides new and effective vaccines which can be serologically discriminated

from field-viruses and old-type vaccines. Such new vaccines, called NDV marker vaccines, provide the fullest possible immunological spectrum of antigenically relevant NDV epitopes, and yet are serologically distinct from wild-type NDV by applying accompanying diagnostic methods and kits.

The invention provides a method for distinguishing unvaccinated animals or animals vaccinated with a NDV vaccine according to the invention from animals infected with wild-type NDV or vaccinated with an unmodified mesogenic or lentogenic NDV-vaccine strain comprising taking a least one sample (such as serum, blood, eggs or eye fluid) from said animal and determining in said sample the presence of antibodies directed against an immunodominant epitope or marker expressed by said wild-type or unmodified NDV but not by a vaccine according to the invention.

The invention provides a method wherein said antibodies are directed against the HN or F protein of NDV, for example a hybrid protein as described in the experimental part as this description.The invention provides for example a diagnostic method wherein said animal is selected from the group composed of poultry, preferably of chickens.

The invention also provides a diagnostic kit for use in a method to serologically distinguish between animals.
In one embodiment of the invention, a simple and rapid haemagglutination-inhibition (HI) test is used to distinguish between vaccinated animals and infected animals. Animals vaccinated with a marker vaccine in which the complete globular head of HN of NDV has been replaced with the corresponding part of HN of another serotype will not induce antibodies to HN of NDV and therefore will not inhibit haemagglutination of erythocytes by NDV virions.

By using marker vaccine virions in the HI test, antibodies against the hybrid HN protein is detected and may used as a measure for the efficacy of vaccination. As an alternative, an ELISA that detects antibodies against the F protein of NDV is used to measure the efficacy of vaccination.